キウイフルーツACC合成酵素の全翻訳領域を含むcDNAの単離とその大腸菌中での活性発現および外果皮におけるACC合成酵素遺伝子の特異的な発現

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Isolation of a cDNA Encoding Active Protein for Kiwifruit ACC Synthase and its Specific Expression in the Outer Pericarp

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Summary

ACC (1-aminocyclopropane-1-carboxylate) synthase cDNA (*AD-ACS1*), isolated from kiwifruit 'Hayward', was expressed as a fusion protein coupled with glutathione S-transferase (GST) in *Escherichia coli*. The fusion protein exhibited ACC synthase activity. Northern blot analyses showed that the transcripts hybridized with *AD-ACS1* accumulated only in the outer pericarp after a 24-hr ethylene treatment.

Key Words: ACC synthase, ACC oxidase, ethylene, fruit ripening, gene expression.

Introduction

In the process of investigating ethylene metabolism in kiwifruit, ACC synthase cDNAs were isolated (Ikoma et al., 1995; Whittaker et al., 1997). However, these cDNAs did not contain the entire coding region for ACC synthase. Whether these cDNAs truly encode enzymatically active protein for ACC synthase has not been confirmed. When the transcript levels for ACC synthase and ACC oxidase were analyzed in kiwifruit, an increase in ethylene production during fruit ripening paralleled the accumulation of ACC synthase transcripts (Whittaker et al., 1997). In melon fruit, transcript accumulations for ACC synthase and ACC oxidase occurred near or at the placental tissue at the initial stage of ripening (Yamamoto et al., 1995). The transcript levels among different tissues in the kiwifruit have not been compared. In this study, cDNA, containing the entire coding region for ACC synthase, was cloned. It expressed itself as a fusion protein, coupled with GST in *E. coli* which confirms that the cDNA encodes an active protein for ACC synthase. The levels of transcripts for ACC synthase and ACC oxidase were compared among different fruit tissues to understand ethylene metabolism in kiwifruit during its ripening stage.

Materials and Methods

Kiwifruit [*Actinidia deliciosa* (A. Chev.) C. F. Liang et A. R. Ferguson var. deliciosa cv. Hayward] were harvested from a commercial orchard in Shizuoka Prefecture early in November. The fruit were placed in a chamber through which 1000 ppm ethylene in air was passed at 12 liter · hr^{-1} for 24 hr at 20 ℃, and then transferred to a 20 ℃ incubator with air ventilation. Ethylene production from individual fruit was measured as described previously (Ikoma et al., 1998). To construct a cDNA library, the outer pericarp tissue was sampled at the active ethylene-producing stage. For Northern blot analyses, transverse fruit slices (1 cm thick) were prepared at the equatorial part of the fruit; the slices were divided into four sections: the outer region of the pericarp, including the epidermis; the mid-section of the pericarp; the inner pericarp, including the locules and seeds; and the white columella. The total RNA was isolated as previously reported (Ikoma et al., 1996). Poly(A)$^+$ RNA was purified, using an oligo(dT)-cellulose column from the total RNA.

From 3 μg of poly(A)$^+$ RNA, a cDNA library was established as described previously (Ikoma et al., 1996). DNA fragments containing previously cloned cDNA (305 bp long cDNA encoding part of the coding region for ACC synthase; DDBJ accession number AB005722, Ikoma et al., 1995) were used as a probe after labeling them by the ECL direct prime labeling and detection system (Amersham). Positive clones were screened by a plaque hybridization method, according to the protocol in the system (Amersham). A cDNA (*AD-ACS1*), subcloned into the Bluescript plasmid, was sequenced.

To express *AD-ACS1* in *E. coli* cells, *AD-ACS1* was subcloned into pGEX-4T-1 (Pharmacia) pre-digested with *Bam*H I and *Xho* I. *E. coli* (XL1-blue) cells were transformed with pGEX or pGEX inserted with *AD-ACS1* and were cultured in a 2xYT medium supplemented with 100 μg · ml^{-1} ampicillin and 0.1 mM IPTG (isopropyl β-D-thiogalactoside) at 27 ℃. After centrifugation, cells were suspended in a saline phosphate buffer containing 0.1% Triton X-100, and sonicated. Fusion protein (*AD-ACS1* protein, coupled with GST) or GST, was purified by affinity chromatography using

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Glutathione Sepharose 4B (Pharmacia); the proteins were then gel-filtrated with 50 mM EPPS buffer (pH 8.5), using PD-10 column (Pharmacia). ACC synthase activities of the proteins (50 μg) were assayed in a reaction mixture, containing 50 mM EPPS buffer (pH 8.5), 10 μM *S*-adenosyl-*L*-methionine (SAM), and 10 μM pyridoxal phosphate (PLP) in a total volume of 1 ml. The succeeding steps for this assay followed the method previously reported by Kasai et al. (1996). SDS/PAGE was carried out, using a 12.5% acrylamide gel. Protein concentration was determined by the method of Bradford (1976).

For Northern blot analyses, *AD-ACS1* (isolated in this study, DDBJ accession No. AB007449) and *AD-ACO1* (DDBJ accession No. AB003514) were used as probes after labeling them with digoxygenin (Boehringer Mannheim). Twenty μg of total RNA from each sample was separated on a 1.2 % agarose-denaturing formaldehyde gel, then transferred to a nylon membrane. The succeeding steps for Northern blot analyses followed the method previously reported by Ikoma et al. (1998).

Results and Discussion

Complementary DNA (1662 bp; *AD-ACS1*) contained a 467 amino acid open reading frame (52.6 kDa; pI 7.8), which began with the ATG codon nearest the 5' end, accompanying a 42 bp 5'-untranslated and a 216 bp 3'-untranslated region (the nucleotide and amino acid sequences are accessible from DDBJ, GenBank and EMBL under the accession No. AB007449). The nucleotide sequence in the coding region was 73.5%, and 71.8%, identical to those of *MAUACCSYN* (Lay-Yee and Knighton, 1995) and *CUCACCA* (Nakagawa et al., 1991), respectively. The deduced amino acid sequence was nearly homologous with the sequence of these ACC synthase genes 75.5%, and 77.9%, respectively, conserving the SAM- and PLP-binding site (SLSKDLGLPGFR; Theologis, 1992). The sequence at the N-terminus in *AD-ACS1* (MKLLSRKAACNSHGQ) resembled that in *CUCACCA* (MKMLSTKATCNSHGQ; Nakagawa et al., 1991). The derived molecular mass of *AD-ACS1* (52.6 kDa) was almost the same as that of *MAUACCSYN* (53.2 kDa; Lay-Yee and Knighton, 1995). These results indicate that *AD-ACS1* contained the entire coding region for ACC synthase. Phylogenetic analysis on the basis of deduced amino acid sequences showed that many auxin-inducible ACC synthase genes from different plants belong to one major lineage of the phylogenetic tree (Liang et al., 1992). *AD-ACS1* was classfied into the branch accompanying many auxin-inducible genes (data not shown).

Fusion protein and GST were successfully purified by affinity chromatography (Fig 1). The molecular mass of the fusion protein measured by SDS-PAGE was 80.0 kDa, consistent with that calculated from the nucleotide sequence (81.4 kDa). The fusion protein contains a protease-recognition site where thrombin can cleave

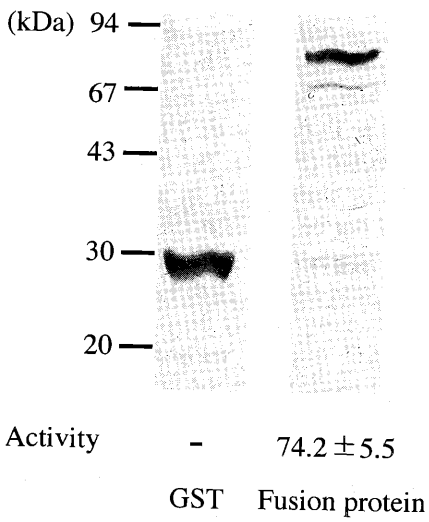

Fig. 1. SDS/PAGE of GST and recombinant ACC synthase expressed as a fusion protein coupled with GST, and ACC synthase activity [nmol ACC · hr^{-1} · $(mg\ protein)^{-1}$] of these proteins. Fifty ng of protein which was loaded onto each lane, became visible with a silver stain.

AD-ACS1 protein from GST. However, in this study the cleavage could not be achieved satisfactorily. To detect enzymatic activity, the fusion protein was used without the cleavage. The fusion protein exhibited ACC synthase activity, whereas GST did not (Fig. 1). These results indicate that the protein derived from *AD-ACS1* was an enzymatically active protein for ACC synthase.

During the ethylene treatment, the transcripts for ACC oxidase rapidly accumulated in every section (Fig. 2). On the other hand, no transcript for ACC synthase was detected in every section before and during the ethylene

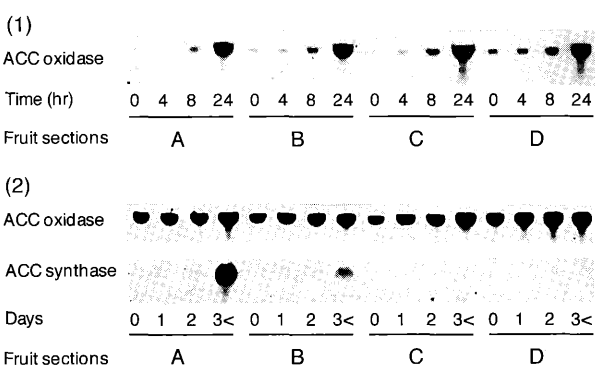

Fig. 2. Northern blot analyses for ACC synthase and ACC oxidase during (1) and after a 24-hr ethylene treatment (2). Fruit Section A, outer region of the pericarp, including the epidermis; Section B, mid-region of the pericarp; Section C, the inner region of the pericarp, including the locules and seeds; Section D, the white columella. For preparation of each sample, sections from three fruit were combined.

treatment (data not shown). Ethylene production and the transcripts were still not detectable for two days after the ethylene treatment. Over three days after the treatment, the fruit produced noticeable ethylene at the rate of 23.5 $\mu l \cdot kg^{-1} \cdot hr^{-1}$ (average of 3 fruit) and the transcripts could be detected (Fig. 2). The level of the transcripts in section A was higher than in section B, but the transcripts could not be detected in sections C and D at this time. The undetectable levels of transcripts for ACC synthase in these tissues must not be due to incomplete penetration of exogenously treated ethylene, because the levels of ACC oxidase transcripts rapidly increased, even in these tissues after the ethylene treatment. Thus, we conclude that the *AD-ACS1* gene is specifically expressed in the outer pericarp after ethylene treatment.

In melon fruit, transcripts for ACC synthase and ACC oxidase were detected initially near or at the placental tissue during the initial stage of ripening (Yamamoto et al., 1995). In kiwifruit, transcripts for ACC synthase were not detected near the seeds even at the actively ethylene-producing stage. We have already shown that seeds or tissues near seeds may play an important role in the ethylene biosynthesis of kiwifruit, by using parthenocarpic kiwifruit in which the ethylene production was reduced and delayed (Ikoma et al., 1998). In kiwifruit, another type of ACC synthase transcripts, which cannot hybridize with *AD-ACS1*, may accumulate in the inner pericarp and columella.

Literature Cited

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72: 248-254.

Ikoma, Y., M. Yano and K. Ogawa. 1995. Cloning and expression of genes encoding ACC synthase in kiwifruit. Acta Hort. 398: 179-186.

Ikoma, Y., M. Yano, K. Ogawa, T. Yoshioka, Z. C. Xu, S. Hisada, M. Omura and T. Moriguchi. 1996. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. J. Japan. Soc. Hort. Sci. 64: 809-814.

Ikoma, Y., M. Yano, Z. C. Xu and K. Ogawa. 1998. Reduction in ethylene synthesis in parthenocarpic *Actinidia deliciosa* fruit induced by *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea. Postharvest Biol. Technol. 13: 121-129.

Kasai, Y., M. Kato and H. Hyodo. 1996. Ethylene biosynthesis and its involvement in senescence of broccoli florets. J. Japan. Soc. Hort. Sci. 65: 185-191.

Lay-Yee, M. and M. L. Knighton. 1995. A full-length cDNA encoding 1-aminocyclopropane-1-carboxylate synthases from apple. Plant Physiol. 107: 1017-1018.

Liang, X., S. Abel, J. A. Keller, N. E. Shen and A. Theologis. 1992. The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 89: 11046-11050.

Nakagawa, N., H. Mori, K. Yamazaki and H. Imaseki. 1991. Cloning of a complementary DNA for auxin-induced 1-aminocyclopropane-1-carboxylate synthase and differential expression of the gene by auxin and wounding. Plant Cell Physiol. 32: 1153-1163.

Theologis, A. 1992. One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell 70: 181-184.

Whittaker, D. J., G. S. Smith and R. C. Gardner. 1997. Expression of ethylene biosynthetic genes in *Actinidia chinensis* fruit. Plant Mol. Biol. 34: 45-55.

Yamamoto, M., T. Miki, Y. Ishiki, K. Fujinami, Y. Yanagisawa, H. Nakagawa, N. Ogura, T. Hirabayashi and T. Sato. 1995. The synthesis of ethylene in melon fruit during the early stage of ripening. Plant Cell Physiol. 36: 591-596.

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摘　要

キウイフルーツから，ACC (1-aminocyclopropane-1-carboxylate)合成酵素をコードするcDNA (*AD-ACS1*)を単離し，大腸菌内でgluthathione S-transferaseとの融合タンパク質として発現させた．この融合タンパク質はACC合成酵素活性を示した．ノーザンブロット解析により果実部位間で転写産物レベルを比較したところ，*AD-ACS1*プローブとハイブリダイゼーションするACC合成酵素遺伝子の転写産物は外果皮においてのみ集積が認められた．